Cadmium-Saturation Technique for Measuring Metallothionein in Brook Trout

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Abstract. — A cadmium-saturation technique for quantifying metallothionein (MT) in mammalian tissues was evaluated for use in fish tissue. We administered 3 mg ^{109}cadmium/kg body weight by intraperitoneal injection over a 5-d period to adult brook trout *Salvelinus fontinalis* to induce MT in liver and kidney tissues. The cadmium-saturation technique was modified so the amount of cadmium bound to unsaturated and cadmium-saturated MT could be measured. The method gave precise measurements of MT concentrations when aliquots of liver supernatant, which were analyzed separately, were quantified by atomic absorption or radiometric measurements. Two to four times more cadmium and MT concentrated in the liver of treated fish than in the kidney. Intraperitoneal administration of cadmium completely displaced copper and zinc from MT in liver of treated fish; cadmium concentrations in liver determined by the quantitation of cadmium-saturated MT and of unsaturated MT were identical. However, exposure of brook trout to cadmium in water did not result in the complete saturation of MT with cadmium. Concentrations of MT and mortality were significantly increased in fish exposed to 5 μg Cd/L or more for 30 d.

Metallothionein (MT) is a low-molecular-weight, cysteine-rich protein that is important for the transport and storage of the essential heavy metals copper and zinc, and may play a part in the detoxification of cadmium and mercury (Kagi and Nordberg 1979). Interest in the detoxification role of MT in fisheries research and aquatic toxicology has increased because of increasing contamination of aquatic habitats by metals. Measurement of MT

has been proposed as a biological indicator of metal stress in humans (Shaikh and Hirayama 1979) and aquatic organisms (Olafson et al. 1979).

In fish, MT characteristically binds 7 g-atoms of a metal such as cadmium per mole of protein (Olsson and Haux 1985; Kay et al. 1986), although 8 g-atoms per mole of protein were reported for one form of MT in rainbow trout *Salmo gairdneri* (Olsson and Haux 1985) and 9 and 10 g-atoms per mole of protein were reported for skipjack tuna *Euthynnus* (=*Katsuwonus*) *pelamis* (Takeda and Shimizu 1982). Saturating MT with respect to one metal and then quantifying that metal would thus result in the indirect quantifi-

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cation of MT. A metal-saturation method for measuring MT described by Piotrowski et al. (1973) was based on the high affinity of MT for mercury at low pH, and the stability and solubility of MT in 5% trichloroacetic acid. Zelazowski and Piotrowski (1977) improved the technique by precipitating MT with 10% tannic acid, which reduced the interference produced by low-molecular-weight mercury-binding substances such as glutathione. Chen and Ganther (1975) quantified MT concentrations by saturating the MT-containing soluble fraction of homogenates with ^{109}cadmium, then isolating MT by gel permeation, and determining the amount of bound ^{109}cadmium. Onosaka et al. (1978) reported a method for quantifying MT by saturating the MT-containing soluble fraction of homogenates with ^{109}cadmium and then removing excess cadmium with guinea pig blood hemolysate, which was in turn removed by heat denaturation and centrifugation. This technique was modified by Eaton and Toal (1982, 1983) to detect quantities of MT as low as 40 ng. This cadmium-saturation technique has been used extensively in studies of mammals but not of fish.

We evaluated the cadmium-saturation method of Eaton and Toal (1982, 1983) for quantifying MT concentrations in fish for use in toxicity studies when large numbers of test animals are analyzed. We administered cadmium to brook trout *Salvelinus fontinalis* by intraperitoneal injection and by water exposure. Cadmium was chosen as the metal stress in these experiments because it induces thionein synthesis and MT formation in fish (Noel-Lambot et al. 1978; Kito et al. 1982b). Cadmium exerts its toxic effect in organisms by displacing copper and zinc in metalloenzymes, which results in a dysfunction by changing the three-dimensional conformation of the enzymes (Friedberg 1974). Recent studies of cadmium toxicity to fish have reported altered enzyme and lysosome functions (Versteeg and Giesy 1986), altered electrolyte and water balance in plasma and urine (Giles 1984), and histopathological changes in proximal renal tubules (Forlin et al. 1986). Toxic effects of cadmium in fish occur at concentrations as low as 0.4 to 0.5 μg/L (Canton and Slooff 1982; Rombough and Garside 1982).

Methods

Brook trout were maintained at the National Fisheries Contaminant Research Center in well water (pH, 7.2; alkalinity, 237 mg/L; hardness, 272 mg/L; temperature, 15°C) and allowed to feed on Rangens® commercial diet ad libitum before

and during the experiments.[2] Fish were raised from eyed eggs obtained from Big Spring Trout hatchery, Lewistown, Montana. Two experiments involving (a) injected and (b) waterborne cadmium were conducted to evaluate the measurement of MT as an indicator of metal contaminant stress.

Exposure to cadmium by injection. — Brook trout were injected with cadmium to induce synthesis of large quantities of MT. Two 1-year-old brook trout (weight about 900 g) were anesthetized with tricaine, weighed, and injected intraperitoneally with carrier-free ^{109}cadmium that had been combined with cadmium chloride and dissolved in 0.9% normal saline to give a specific activity of 76,000 counts/min per mg cadmium in 1 mL. Fish were injected with 0.5 mg Cd/kg on day 1, 1.0 mg Cd/kg on day 3, and 1.5 mg Cd/kg on day 5, and were held in a 700-L tank. Two additional 1-year-old brook trout, injected with similar volumes of 0.9% normal saline on days 1, 3, and 5 to stimulate the stress of repeated handling and injection, were maintained in a 700-L tank separate from fish injected with ^{109}cadmium. On day 8, all fish were killed and livers and kidneys were removed immediately, weighed individually, and then homogenized individually in ice-cold 0.01 M tris–HCl buffer (pH 8.1) with 0.01% sodium azide in the ratio of 1 g tissue per 7 mL buffer. The homogenate was denatured by heating in a water bath at 100°C for 3 min to eliminate protease activity. Homogenates were centrifuged at 10,000 × gravity for 20 min at 4°C to remove particulate matter. The initial heat denaturation and centrifugation steps, after tissue homogenation, removed some of the high-molecular-weight proteins but did not remove MT or other low-molecular-weight proteins that are heat stable. The supernatant was then centrifuged at 100,000 × gravity for 60 min at 4°C to separate the cytoplasmic fraction from other cellular components. The supernatant from the 100,000 × gravity centrifugation was used in further isolation and characterization procedures.

Cadmium was quantified in three fractions: 100,000 × gravity supernatant, saturated MT, and unsaturated MT. The concentration of cadmium in the 100,000 × gravity supernatant was determined by digestion of an aliquot of supernatant in concentrated nitric acid for 16 h at 70°C and quantification by atomic absorption and graphite

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[2] Reference to trade names, commercial products, or manufacturers does not imply or constitute government endorsement or recommendation for use.

furnace techniques on an instrument equipped with deuterium background correction. The fraction of the tissue supernatant termed saturated MT was quantified indirectly by the method of Eaton and Toal (1982, 1983) and involved six steps: (1) saturation of MT in an aliquot of 100,000 × gravity supernatant by addition of excess cadmium at 3.56 nmol and incubation at room temperature for 10 min; (2) removal of nonmetallothionein-bound cadmium by addition of 2% hemoglobin and incubation at room temperature for 10 min; (3) denaturation of the cadmium–hemoglobin complex by incubation in a water bath at 100°C for 3 min, followed by rapid cooling on ice for 3 min; (4) centrifugation at 10,000 × gravity for 5 min; (5) digestion of an aliquot of supernatant containing MT-bound cadmium in concentrated nitric acid for 16 h at 70°C; and (6) quantitation of this cadmium by atomic absorption or radiometric measurement with a scintillation counter. Samples quantified by radiometric measurement were saturated through use of a mixture of [109]cadmium and cadmium chloride in distilled water with a specific activity of 250,000 counts/min per μg cadmium in 1 mL. All scintillation counts were corrected for background levels of [109]cadmium present in samples from the intraperitoneal injection. Samples quantified by atomic absorption measurement were saturated with nonradiolabeled cadmium chloride dissolved in distilled water. MT was calculated by the equation:

$$\mu g \text{ MT/g tissue}$$
$$= [(\text{mg Cd/L in sample})$$
$$\times (1 \text{ nmol Cd}/112.4 \text{ ng Cd})$$
$$\times (1 \text{ nmol MT}/7 \text{ nmol Cd})$$
$$\times (6,000 \text{ ng MT}/1 \text{ nmol MT})]/g \text{ tissue.}$$

The quantity of cadmium bound to MT in its natural state was termed unsaturated MT and was determined by a modification of Eaton and Toal's (1982) method. Two deviations were made from the Eaton and Toal (1982) procedure described earlier: first, the binding sites on MT were not saturated with excess cadmium because only MT-bound cadmium was to be quantified; second, the concentration of hemoglobin added to remove free cadmium and aid in coagulating low-molecular-weight proteins was 1% instead of 2%. We conducted a test with 0, 0.4, 0.8, 1.2, 1.6, and 2.0% hemoglobin solutions to determine the lowest concentration that effectively removed free cadmium (nonmetallothionein-bound) from solution without removing MT-bound cadmium.

Another aspect of the Eaton and Toal method

evaluated was the cadmium-binding capacity of MT in various concentrations of homogenized tissue. We evaluated their method by measuring the binding capacity of 3.56 nmol cadmium in serially diluted samples of liver homogenate from fish number 1 injected with [109]cadmium. Two sets of samples were analyzed separately by the cadmium-saturation technique; one set was quantified by atomic absorption measurement and the other by radiometric measurement.

Exposure to waterborne cadmium. —A chronic toxicity study was conducted with brook trout (average 10 g; age 3–4 months) continuously exposed to concentrations of 0, 1.0, 2.1, 5.0, 10.5, and 19.1 μg Cd/L as cadmium chloride for 30 d in an intermittent-flow diluter (Mount and Brungs 1967). Cadmium chloride dissolved in pure water, and a mixture of sulfuric acid and nitric acid (3:1, volume : volume), were metered into the diluter with an automatic pipette; the cadmium was added to the toxicant mixing chambers, and the acid mixture was added to the flow-splitting chambers. The diluter combined cadmium and the acid mixture with reconstituted low-alkalinity–low-hardness water in a series of variably sized glass boxes to create five cadmium concentrations and one control with equal total volumes. Various concentrations of cadmium in reconstituted water were delivered by glass tubes to flow-splitting chambers (Benoit and Puglisi 1973), which thoroughly mixed cadmium and the acid mixture with reconstituted water and divided equal volumes of each cadmium concentration for delivery by glass tubes to duplicated exposure aquaria (Cleveland et al. 1982). The diluter was adjusted to cycle every 15 min and delivered 1 L of exposure water per cycle to each of 12 exposure aquaria.

The acid mixture and reconstituted low-alkalinity–low-hardness water were incorporated into the experimental design of this study to enhance the availability of cadmium to fish and to stimulate acidified lake water conditions in the northeastern USA. The reconstituted water was created by blending high-purity water from a water softener—reverse osmosis—deionizer water treatment system with well water. A photoperiod of 16 h light and 8 h dark was controlled by a timed, artificial lighting system (Drummond and Dawson 1970). Water quality characteristics measured during the study approximated: pH, 6.2; alkalinity, 2 mg/L; hardness, 6.6 mg/L; conductivity, 25 μs/cm; dissolved oxygen, >8.5 mg/L; and temperature, 12°C. These variables were measured twice weekly in all aquaria, except pH was mea-

sured daily. The pH, conductivity, and hardness of exposure water were determined according to standard procedures (APHA et al. 1975). Alkalinity was determined by the Gran Plot method (Stumm and Morgan 1981).

We placed 25 fish in each of 12 exposure aquaria and acclimated them first from well water to the reconstituted low-alkalinity–low-hardness water over a 48-h period and then from pH 7.2 to pH 6.2 over an additional 48-h period before cadmium was added to the exposure water. Mortality was recorded daily and dead fish were removed from the aquaria.

After 30 d of exposure, two pooled samples of four fish each were randomly sampled from each duplicate concentration of cadmium. Fish were anesthetized with tricaine and weighed (grams). Livers were removed and weighed individually, then combined into pooled samples and homogenized in 14 mL ice-cold 0.01 M tris–HCl buffer (pH 8.1) with 0.01% sodium azide. Homogenates were heat-denatured and centrifuged as described for the injection study. Cadmium was quantified, as described for the injection study, in three fractions: 100,000 × gravity supernatant, unsaturated MT, and saturated MT fractions.

As an additional check on the cadmium-saturation method, we measured MT concentrations in two sets of liver supernatants of fish exposed to waterborne cadmium. One set was measured by atomic absorption and, 3 weeks later, the other set was separately analyzed and measured by radiometry.

Statistical analysis.—Statistical analyses were performed with Statistical Analysis System programs. The percent mortalities of fish in the waterborne study were analyzed by one-way analysis of variance on arcsine-transformed values. Toxicant effects on various cadmium-containing tissue fractions (100,000 × gravity supernatant, unsaturated MT, and saturated MT) were determined by one-way analysis of variance. These variables were first examined by regression analysis of their means, standard deviations, and variances to determine if transformation was needed to normalize data before statistical analysis. Logarithmic transformations were applied to all cadmium-containing tissue fractions before analysis because correlations (r) between means, standard deviations, and variances were greater than 0.40. Treatment means were compared by multiple-means-comparison tests (least significant difference; Snedecor and Cochran 1967). Linear regression analysis was used to determine the relation be-
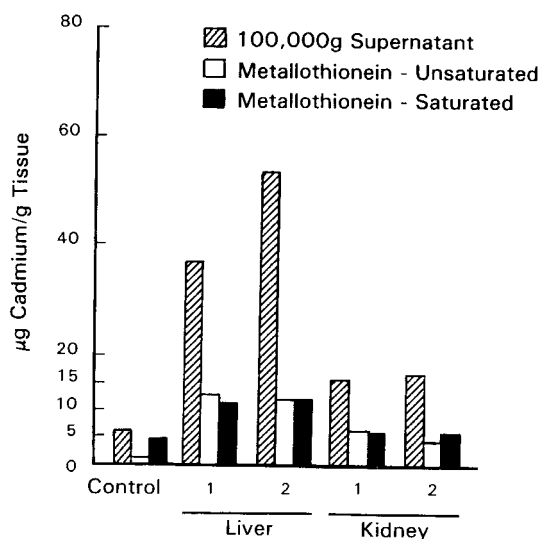

FIGURE 1.—Cadmium in various fractions of liver and kidney tissues from two brook trout (1, 2) injected with [109]cadmium; 100,000g = 100,000 × gravity centrifugation level.

tween atomic absorption and radiometric measurements in various supernatant fractions.

Results

Evaluation of Cadmium-Saturation Method

Cadmium concentrations increased significantly in the 100,000 × gravity fraction of liver and kidney tissues of the cadmium-injected fish (Figure 1). Livers of fish accumulated about 26% of the total [109]cadmium injected. During the centrifugation steps, about 0.6% of the total cadmium in the liver homogenate was in the particulate pellet. The amount of cadmium present in liver and kidney of control fish was uniformly low and, therefore, control fish were combined as a single group in Figure 1.

In the test to determine the lowest concentration of hemoglobin that effectively removed the free cadmium (nonmetallothionein-bound) from solution, we found hemoglobin concentrations between 0.8 and 2% removed cadmium that was not bound to MT (Figure 2). Thus, to conduct the unsaturated MT procedure, we chose to use 1% hemoglobin for the removal of free cadmium.

The cadmium-binding capacity of MT in various concentrations of homogenized tissue showed a linear response between the amount of cadmium bound and tissue concentration (Figure 3). About 76% of the added cadmium was bound to the highest tissue concentration tested (142 mg/mL)
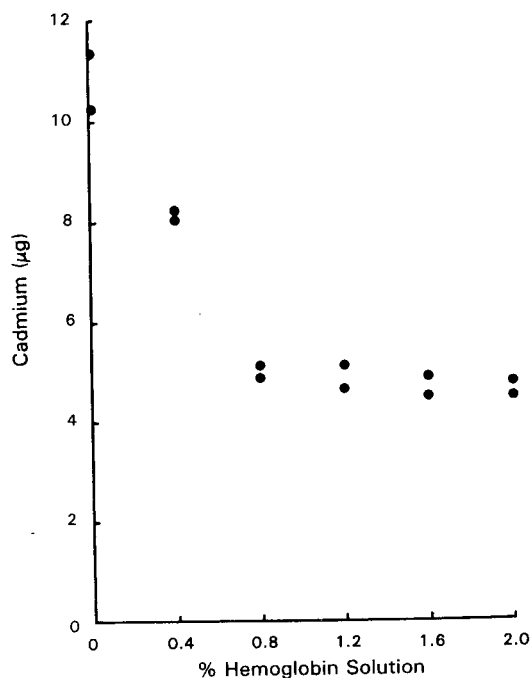
FIGURE 2.—Relation between percent hemoglobin solution used to remove free cadmium and the amount of metallothionein-bound cadmium remaining in solution.

whereas only 14% was bound in the lowest (4.5 mg/mL). In two sets of serially diluted aliquots of one liver supernatant analyzed separately by the cadmium-saturated MT method, the ratio of atomic absorption measurement to radiometric measurement was 1.05:1 (Figure 3). Concentrations of homogenized tissue of fish exposed to waterborne cadmium were below those present in undiluted homogenates from fish injected with [109]cadmium (142 mg tissue/mL). Therefore, the technique was assumed to give a linear response between tissue concentration in the homogenate and MT quantitation in samples from the waterborne cadmium study.

Measurement of unsaturated and saturated MT in the liver and kidney of cadmium-injected fish showed that MT was completely saturated with cadmium from the injection because cadmium concentrations were nearly identical in MT quantitations (Figure 1). However, in control fish, the amount of cadmium bound to MT in its natural, unsaturated condition was less than its potential cadmium-binding capacity, i.e., saturated MT. The concentration of MT was about two times greater in liver of treated fish than in kidney.

In our last check on the cadmium-saturation method, we measured MT concentrations in two aliquots of liver supernatants of fish exposed to waterborne cadmium. The aliquots were analyzed separately 3 weeks apart but both quantitations gave similar results and the ratio of atomic absorption measurement to radiometric measurement was 0.96:1 for the 23 samples analyzed. The mean values for each measurement for fish within a treatment are given in Table 1.

Exposure to Waterborne Cadmium

Mortality was significantly increased after 30 d by exposure to 5.0 μg Cd/L or more (Table 2); however, there was no dose–response relationship in mortality of fish exposed to cadmium at 5.0, 10.5, and 19.1 μg Cd/L. Fish growth, as measured by weight, was not affected by cadmium exposure (Table 1).

After 30 d of exposure, the 100,000 × gravity supernatant of the liver homogenate contained cadmium concentrations approximately four to five times higher in exposed fish than in the controls (Table 1). However, the amount of cadmium present was not different among livers of fish exposed to different concentrations of cadmium. Concentrations of MT in the liver were significantly elevated in exposure concentrations of 5.0 μg Cd/L and greater, which coincided with significant increases in mortality. These concentrations did not, however, increase in a dose–response pattern with increasing cadmium exposure. The lack of a dose-dependent relation between cadmium exposure concentration and cadmium in either the 100,000 × gravity supernatant or saturated MT indicated that cadmium uptake in the liver seemingly had reached an equilibrium between uptake and excretion. The quantity of cadmium bound to MT in an unsaturated condition was significantly elevated in cadmium exposure concentrations 1.0 μg Cd/L and higher, and also was highly correlated with cadmium exposure concentration ($r = 0.83$). This increase indicated that cadmium was bound to MT in direct proportion to the amount of cadmium exposure. Cadmium was not bound in sufficient quantities, however, to increase MT concentrations in fish exposed to cadmium concentrations less than 5.0 μg Cd/L.

In our experiment, about 8% of the cadmium in the liver of treated fish was present in the unsaturated MT fraction, compared with the amount in the 100,000 × gravity supernatant (Table 1). Concentrations of cadmium in the unsaturated MT fraction were low even though the quantitation of saturated MT indicated that a large binding ca-
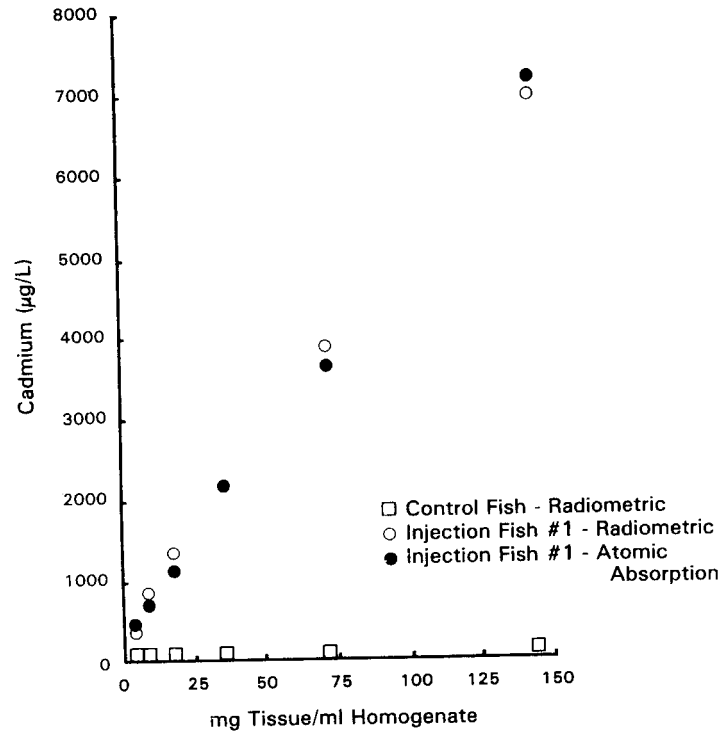
FIGURE 3.—Relation between different tissue concentrations of liver homogenate and the amount of bound cadmium, when the metallothionein quantitation technique of Eaton and Toal (1982, 1983) was used. Cadmium in liver homogenate from [109]cadmium-injection fish number 1 was measured by atomic absorption and radiometric techniques, whereas cadmium in liver homogenate from normal saline-injected control fish was measured only by radiometry.

TABLE 1.—Means and (in parentheses) SDs of cadmium concentrations in various liver fractions and weights of brook trout exposed to waterborne cadmium for 30 d. Asterisks denote significant differences from control values ($P \leq 0.05$); MT = metallothionein.

Exposure concentration (μg Cd/L)	Cadmium-containing fraction				MT[a] (μg/g tissue)	MT[b] (μg/g tissue)	Fish weight (g)	Number of pooled samples
	Supernatant (100,000 × gravity) (ng Cd/g tissue)	Free cadmium (ng Cd/g tissue)	Unsaturated MT (ng Cd/g tissue)	Saturated MT (ng Cd/g tissue)				
0	40 (10)	40 (10)	0	1,198 (218)	9.4 (2.2)	8.8 (1.2)	11.3 (1.6)	4
1.0	177* (25)	171* (26)	6* (4)	1,235 (148)	8.4 (0.7)	10.5 (1.6)	12.1 (1.4)	4
2.1	203* (31)	195* (29)	8* (3)	1,494 (337)	11.2 (2.5)	11.6 (2.7)	10.7 (1.2)	4
5.0	175* (35)	162* (28)	13* (8)	1,720* (234)	12.8* (2.1)	13.4* (1.5)	11.8 (2.0)	4
10.5	137* (24)	118* (20)	19* (13)	1,626* (268)	12.0* (2.0)	12.8* (2.1)	13.5 (2.2)	4
19.1	232* (70)	202* (64)	30* (7)	1,912* (385)	14.9* (3.8)	14.3* (2.1)	12.1 (3.1)	3

[a] Atomic absorption quantitation.
[b] Radiometry quantitation.

TABLE 2.—Percent mortality of brook trout exposed to waterborne cadmium. Asterisks denote significant differences from control values ($P \leq 0.05$); $N = 50$.

Exposure concentrations (µg Cd/L)	Days of exposure		
	7	15	30
0, 1.0, 2.1	0	0	0
5.0	2.0	4.5	27.2*
10.5	8.0	15.2*	35.1*
19.1	22.0*	26.9*	33.1*

pacity was potentially available. Measurement of saturated MT in liver showed that the cadmium-binding potential of MT was about nine times greater than the amount of cadmium present in the 100,000 × gravity supernatant and 112 times greater than the amount of cadmium bound to unsaturated MT. The cadmium representing the difference in total cadmium concentration between the amount present in the 100,000 × gravity supernatant and the unsaturated MT fraction, termed free cadmium, probably was bound to nonmetallothionein proteins or enzymes and therefore exerted a sublethal influence. The amount of free cadmium in the liver was elevated above control concentrations in fish from all exposures and represented about 92% of the cadmium present in the 100,000 × gravity supernatant.

Discussion

Evaluation of Cadmium-Saturation Method

The complete saturation of MT in liver and kidney with cadmium in our injection study is similar to findings reported by Winge et al. (1978) and Day et al. (1984) for rats injected with cadmium and examined after 2 to 3 h. Kito et al. (1982a) reported that MT in liver of common carp *Cyprinus carpio* injected with 2 mg Cd/kg body weight daily for 6 d and examined 24 h later was nearly saturated with cadmium but still had minor amounts of zinc bound to it. However, Ley et al. (1983) reported that MT was not saturated with zinc in rainbow trout injected with 10 mg zinc/kg daily for 2 d but, rather, had substantial amounts of copper bound to it. Their results are not surprising because the binding affinity of MT for zinc is less than that for copper. Similar to our results, Woodworth et al. (1983) reported greater amounts of cadmium were present in liver than in kidney of cadmium-injected fish.

In our study, we selected 1% hemoglobin to remove free cadmium and aid in coagulating low-molecular-weight proteins in the unsaturated MT method. Eaton and Toal (1982) examined the ef-

ficiency of cadmium removal in tissue homogenates containing MT by repeated hemoglobin additions and reported that as the number of hemoglobin additions increased, the estimate of MT decreased. They attributed this phenomenon to an apparent "stripping" of cadmium from MT after each hemoglobin addition. Furthermore, they proposed that if there is an equilibrium established between free and MT-bound cadmium, removal of free cadmium upon hemoglobin precipitation would result in dissociation of the cadmium–MT complex to reestablish the equilibrium. In our study, measurement of the amount of cadmium bound to MT in its natural state required that we determine the lowest concentration of hemoglobin that effectively removed free cadmium and, at the same time, reduced the possibility of underestimating MT due to the "stripping" phenomenon. In contrast to this phenomenon, Scheuhammer and Cherian (1986) reported that the cadmium content of purified MT from liver of rats injected with cadmium was unaffected by hemoglobin treatment when MT was in an unsaturated condition.

Our evaluation of the relation between cadmium-binding capacity of MT and tissue concentration showed a linear response that was similar to one reported by Eaton and Toal (1982). They reported that their method gave a good estimate of MT concentrations in tissue if 50% or less of the added cadmium was bound to MT in the saturation step, and they recommended dilution of tissue homogenates if a greater percentage of cadmium was bound in undiluted tissue homogenates. In the cadmium saturation step of the procedure, sufficient exogenous cadmium must be incubated with homogenate containing MT to enable the complete saturation of MT with cadmium and the displacement of other metal ions from it. The addition of excess cadmium, however, may disrupt hemoglobin-mediated removal of unbound cadmium during the appropriate step in the procedure if the amount of cadmium added exceeds the binding capacity of the hemoglobin addition. Likewise, tissues with large quantities of MT and, concomitantly, large quantities of protein, may not become completely saturated with cadmium if the quantity of MT is greater than the quantity of cadmium added in the saturation step. Consequently, in the saturation procedure, a balance must be effected between the quantity of MT present in the tissue supernatant (i.e., the tissue concentration of homogenate), the quantity of exogenous cadmium added to saturate MT, and the

amount of hemoglobin needed to remove unbound cadmium. In the cadmium-saturation method, the last two conditions are held constant; therefore, the quantity of MT present in the tissue must be evaluated for its cadmium-binding capacity to determine if dilutions of the tissue homogenate are needed. Consequently, we recommend that preliminary evaluations of this method be made with tissue homogenates of animals stressed by metals known to increase MT concentrations to determine the relation between cadmium-binding capacity and tissue concentration.

Since publication of the modified cadmium-saturation method by Eaton and Toal in 1982–1983, the method has been examined further and found to be a good measure of MT concentrations in tissues. Law and Stillman (1984) examined detailed spectral changes of MT as modified by the cadmium-saturation method and reported the method gave an accurate estimation of MT concentrations when based on the complete saturation of all binding sites on MT with cadmium and removal of excess cadmium by hemolysate treatment. Waalkes et al. (1985) reported that results with the cadmium-saturation method in cadmium- or zinc-treated animals were similar to those with a mercury-saturation method and comparable to results from a radioimmunoassay. However, in animals in which copper and mercury are already bound to MT at relatively high concentrations from a prior exposure, the cadmium-saturation method may underestimate MT concentrations because cadmium would not effectively displace elevated amounts of these MT-bound metals (Eaton 1985). In a recent study, Scheuhammer and Cherian (1986) confirmed that the cadmium-saturation method consistently underestimated the concentration of MT in livers of copper-treated rats. They also compared the cadmium-saturation method with their newly developed silver-saturation method and found excellent agreement in the measurement of MT from various sources not previously exposed to abnormal concentrations of copper.

Exposure to Waterborne Cadmium

In our study, exposure to cadmium concentrations as low as 1 µg/L resulted in a fourfold increase in the amount of cadmium in the liver compared to concentrations in control fish. Application of the unsaturated MT method in our study revealed that only 8% of this cadmium was bound to MT in its natural state and was elevated in all exposure concentrations above those in control

fish. These results are similar to those of Kotsonis and Klaassen (1977), who reported that only 25–39% of the available sites on MT were occupied by cadmium in cadmium-injected rats killed 48 h after treatment. In their study, the cadmium-binding capacity of MT, as measured by a cadmium-saturation technique, was three to four times greater than the amount bound to it from the exposure. Kito et al. (1982b) reported that only a small amount of cadmium was bound to the MT-fraction of the hepatopancreas of common carp exposed to 1 mg Cd/L for 14 d. Visual inspection of their data revealed that the cadmium-binding capacity in exposed fish was about eight times greater than the amount of cadmium bound to it in its natural condition because the MT-fraction of exposed fish contained substantial amounts of zinc and small amounts of copper. Several investigators have reported results similar to these; chronic exposure of fish to cadmium in water results in a mixture of cadmium, zinc, and copper bound to MT (Coombs 1975; Noel-Lambot et al. 1978; Hidalgo et al. 1985). Their results show that MT is not saturated with cadmium under chronic waterborne exposure conditions, which implies that the cadmium-binding capacity of MT is greater than the amount of cadmium bound to it in its natural condition.

Utility of Metallothionein Measurement

Measurement of MT concentrations in aquatic organisms may provide a useful index of exposure to certain heavy metals in the environment (Neff 1985). The metal-saturation method of quantifying MT concentrations is a sensitive method suitable for large numbers of samples. Application of the technique quantifying cadmium bound to MT under saturated and unsaturated conditions to fish populations stressed by metals and control populations would be relatively easy. Fish would have to be collected rapidly and killed, and tissue samples rapidly removed and frozen, to minimize protease activity on MT and other proteins. The two most time-consuming steps in the analysis process would be (1) liver removal, homogenization, and centrifugation (24 samples can be processed in 1 d) and (2) atomic absorption analysis of cadmium concentrations (24 supernatant samples [100,000 × gravity] and 24 unsaturated MT samples can be processed in 1 d). The technique for isolating unsaturated MT is precise and requires little time; 24 samples can be processed in 1–2 h. Free cadmium in the liver of fish could be measured easily by using the techniques described

here, and those measurements would give insight into the health and well-being of fish populations.

The present study was undertaken to evaluate the cadmium-saturation method of Eaton and Toal (1982, 1983) in a low-cadmium exposure study with brook trout. Following this study, we conducted a high-cadmium exposure study with a slightly earlier life stage of brook trout to further evaluate the technique as a biological indicator of cadmium stress on fish; the results of the high-cadmium exposure study are presented in the following paper (Hamilton et al. 1987). The purpose of the high-cadmium study was to create a stronger dose–response relation for mortality and to assess the relation of MT concentrations in liver and kidney to mortality and whole-body residues of cadmium.

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